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14. ABSTRACT

Purpose: Alternative splicing is responsible for producing several products from a single transcript and can cause pathogenic changes in RNA in neurodegenerative disease. This proposal tests the hypothesis that regulation of normal splicing is disrupted in Parkinson's disease (PD). Scope: Experiments are designed to determine splicing products in the brain and blood of experimental MPTP models of PD and the blood of newly diagnosed PD patients, who are not yet on dopamine therapy. The overall goal is to use splice variants as biomarkers to identify individuals at risk for PD. To date, we have identified and quantified alternatively spliced transcripts for several candidate genes in MPTP models of PD. We have also obtained IRB permission to study splicing factors in the blood of newly diagnosed PD patients. Major Findings: Mice treated chronically with MPTP show a shift in the ratio of FosB, RGS9 and Ania6 splice variants in the striatum, 3 days post-treatment. The splicing ratios for AChE and Ania 6 also change in the blood following chronic treatment and, for Ania 6, the changed ratio persists up to 3 weeks after treatment. Progress in the first year includes 4 abstracts, a peer-reviewed publication and an article in preparation.

15. SUBJECT TERMS
Pre-mRNA splicing, MPTP, Parkinson's disease, gene expression, alternative splicing, intron, exon

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INTRODUCTION

An important pathological hallmark of Parkinson's disease is the progressive and selective loss of nigrostriatal dopamine (DA) neurons. The mechanisms underlying the development and progression of this disease is not fully understood, although increasing evidence points to factors such as mitochondrial dysfunction, oxidative damage, excitotoxicity, and inflammation as contributors to cell death (*1-7*). There is no cure for this disease and, to date, no biomarkers to identify susceptible individuals. With new tools, such as those that we have been developing, we will try to identify presymptomatic individuals on the basis of a shift in the ratio of certain splicing variants in the blood. Alternative splicing, one mechanism that regulates gene expression, is responsible for producing several products from a single transcript and can cause pathogenic changes at RNA and protein levels. A few alternative splice products have been identified in experimental parkinsonism and confirmed in Parkinson's disease (PD) patients. Such variants are produced in different proportions in healthy individuals. Changes in gene expression due to various cell stressors during the neurodegenerative process will alter the regulation of pre-mRNA splicing and thereby change the ratio of splice products (*7*). ***We therefore hypothesize that (1) an altered 'ratio' of splice products can be used as a molecular marker of disease pathology, and (2) altered ratios can be identified in the blood of individuals in presymptomatic stages of disease***. Such early detection is increasingly important as new neuroprotective agents are developed and could be used to combat disease in early stages. Furthermore, the elucidation of altered gene products sets the stage for the development of novel therapeutic strategies to target specific splicing isoforms. Discovery of alternative splicing events in the blood and brain of an experimental model of PD, and in the blood of humans, newly diagnosed with PD, form the main objective of this research.

BODY

This is a 3-year project and during this first year, we have made excellent progress towards our goals. We have published one peer-reviewed paper (a review of dysregulation of gene expression in neurodegeneration) and have a second manuscript in preparation. We have also had 4 abstracts accepted for presentation at scientific meetings, including the World Parkinson Congress. The Body of this report will begin with details of the Personnel on the project and the successes and problems of our technical approach. It will then describe our accomplishments and any problems that have arisen for each Objective outlined in the approved Statement of Work. Considerable progress has been made toward the initial goals of Objectives 1 and 3.

Personnel

It is important to note that the personnel for this project had to be hired and/or trained during the initial months of this 3-year project. We began by hiring a Senior Research Investigator. His training was not appropriate and he left the project after 6 months. This hampered our initial progress. Nevertheless, following this period, we were fortunate to hire a Molecular Biologist with extensive experience. Under Dr. Patricia Loomis' able guidance, we have now made excellent progress. In addition, Mrs. Jennifer Jackolin (part-time) and Mrs. Francine Jodelka have joined the team. The key personnel for the 3-year project are now in place. We meet often, and the investigations are close to being on target.

Overall Technical Approach

We originally proposed to carry out our investigations in animal models of PD, either the rat, 6-hydroxydopamine (6-OHDA) model or the mouse, MPTP model. After careful consideration, we decided that the 6-OHDA lesion was inappropriate for the goals of this study. This is because this lesion is invasive and causes an increase in the permeability of the blood brain barrier, which can lead to a functional loss of the catecholamine membrane

pump (*8*). Moreover, the ultrastructural signs of degeneration in the substantia nigral, dopamine cells are similar to those seen after surgical axotomy (*9*), a feature absent in PD. The neurotoxic effects of MPTP more completely model the pathophysiology of PD. In mice and non-human primates, MPTP mimics the action of this toxin in humans (*6, 10-12*). We have therefore elected to treat mice with MPTP, either acutely, which depletes the substantia nigra of approximately 50% of the dopamine cells, or chronically with a low dose MPTP treatment, which can kill as many as 80-90% of the dopaminergic neurons (*13*). We treat male, C57Bl/6 mice that weigh 18-22g at the start of the experiments. For the acute procedure, we inject them with 20mg/kg MPTP (in saline), 4 times, every 2 hours, and euthanized them 3 days later. For the chronic, low-dose procedure, we inject mice twice each week, at 3.5-day intervals, for 5 weeks with MPTP (25 mg/kg, s.c) and probenecid (250 mg/kg, i.p.). Mice are then euthanized either at 3 days or 3 weeks after treatment. Two time periods were selected because we are seeking splicing events that remain stable in the blood over time. Controls for the acute group include mice treated with saline and, for the chronic experiments, mice treated with probenecid or saline. We have yet to treat old (> 12 months of age) mice to establish the contribution of aging to the splicing events. We have encountered no difficulties with either treatment regimen. There is sufficient blood and brain material, except for midbrain where we have to pool tissue from 2 animals, for the experiments.

Quantifying the splice variants has been successful. However, we have had to pursue an alternative approach. Initially we proposed to utilize TaqMan real time RT-PCR to quantify splice variants from the blood and brains of control and MPTP treated mice. In this method the reverse transcription and amplification steps are performed in a PCR machine equipped with sensors to record the reaction progress during each amplification cycle by the detection of the fluorescent reporters. Therein, results are appraised in real time and the need for agarose gel electrophoresis to detect the products of the PCR reactions is eliminated. Preliminary studies using TaqMan fluorescent probes and primers provided evidence that this method would not be useful for our analysis because of the restrictive nature of the design of the primers and probes. Optimal conditions for RT-PCR utilize a ~100 nucleotide sequence within or overlapping the alternatively spliced region of the mRNA. The GC content of this region must be sufficient enough to allow selection of primers (~20-25 nucleotides) and probe (~30 nucleotides) with melting temperatures between 60-70 ^{0}C. The probes must be designed so that there is minimal or no intramolecular complementarity and no complementarity with the primers. As we began to analyze the alternatively spliced regions of various pre-mRNAs we found these requirements of GC content and sequence non-complementarity to be too restrictive. Often the alternatively spliced region is less than 100 nucleotides and it was impossible in many cases to design both primers and probes that would function in a real time RT-PCR reaction. To solve this problem we decided to instead design primers that would flank the alternatively spliced region of the mRNA. Using this paradigm we have the ability in one PCR reaction to amplify both the spliced and non-spliced mRNA. Products of the PCR amplification are detected by agarose gel electrophoresis and quantified using the Kodak EDAS 290 system. Data analysis consists of comparing the amount of splice variant PCR product to non-spliced variant PCR product. Also, both PCR products are in turn compared to the amount of 18S rRNA PCR product. Although this method is semi-quantitative we have been able to generate reproducible, significant data.

Objective 1: To identify abnormal splice variants of genes involved in the development and progression of Parkinsonism in the brain and blood of chronic rodent models of PD.

Currently, there are no studies on changes in pre-mRNA splicing in PD models, partly because until this year, there has been no means of detecting splice variants using microarrays. It is crucial to investigate this area of gene expression since pre-mRNA splicing is an important means of regulation. It is clear that changes in splicing are produced after exposure to stress in many neurodegenerative diseases. Even with the limitations of microarray assays, these have provided an abundance of information on changes in gene expression that occur in animal models of PD (*14-16*). We have focused our search for splice variants primarily on 'stress-response' genes and a few other genes, such as FosB, that may play a role in the development of PD (*17*).

Results:

Mice for splicing studies and HPLC measurements of striatal dopamine and its metabolites, are euthanized with cervical dislocation followed by decapitation; their brains are dissected over ice. This euthanasia method was chosen to minimize the stress to the animal and avoid the massive release of stress hormones. Midbrains from some mice are placed in 3% paraformaldehyde in 0.1M phosphate buffer for a week. These midbrains are then sunk in 20% sucrose and cut on a cryostat. Sections are immunoreacted for tyrosine hydroxylase (in order to visualize the dopaminergic neurons in the substantia nigra) and counterstained for Nissl substance, using cresyl violet. Neurons that survive the MPTP lesion are counted using unbiased stereology. Mice from the chronic studies are also analyzed with a behavioral test carried out the day before they are killed. We can thus correlate changes in splice variants with the loss of striatal dopamine, dopaminergic neurons in the substantia nigra and motor behavior, over time. It is likely that the ratio of splice variants for different transcripts will change over time during the post-treatment period. By taking samples during the first week, we can establish a baseline for splice ratios and then establish later whether these changes persist. In a pilot study, we have examined one splice ratio, ΔFosB/FosB, in both saline and probenecid controls, since probenecid could potentially produce aberrant splice variants. We found no difference between these two different control groups and have therefore elected to use saline-treated animals as the vehicle group in all experiments.

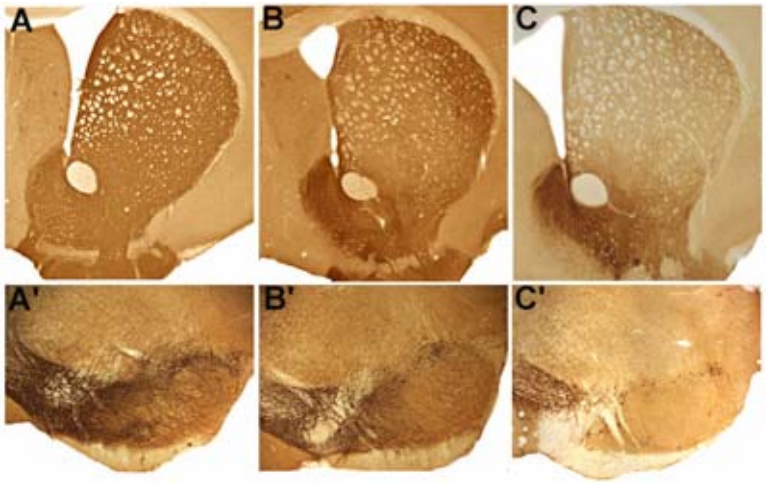


Figure 1. tyrosine hydroxylase immunoreativity in the striatum (A,B,C) and midbrain (A',B',C') of mice treated with vehicle (A,A'), acute MPTP regimen (B,B'), and chronic MPTP procedure (C,C').

Figure 1 illustrates the differences in tyrosine hydroxylase immunoreactivity in the midbrain and forebrain following acute or chronic MPTP treatment. Table 1 shows the findings for dopaminergic neuron cell counts using stereology. As predicted, there is a greater loss of dopaminergic neurons after the chronic treatment. This is also demonstrated by the significantly poorer performance on the grid test for MPTP-treated mice when compared to vehicle controls (Figure 2). Assaying brain tissue levels of dopamine and its metabolites, DOPAC and HVA will be done by high performance liquid chromatography (HPLC, Waters,

Milford, MA) (*13*). This work is being carried out in collaboration with the laboratory of Dr. Un Kang at the University of Chicago. The first assays are being completed in October.

Treatment	Mean Total Number of Neurons	Coefficient of error
Vehicle	9998 +/- 47	0.06
Acute MPTP	6665 +/- 53	0.08
Chronic MPTP	4763 +/- 98	0.09

Table 1. Mean total number (+/- s.e.) of Nissl-stained neurons in the substantia nigra pars compacta of mice treated with vehicle, acute MPTP, or Chronic MPTP (3 weeks post-treatment). Data were collected using the optical fractionator and unbiased stereology.

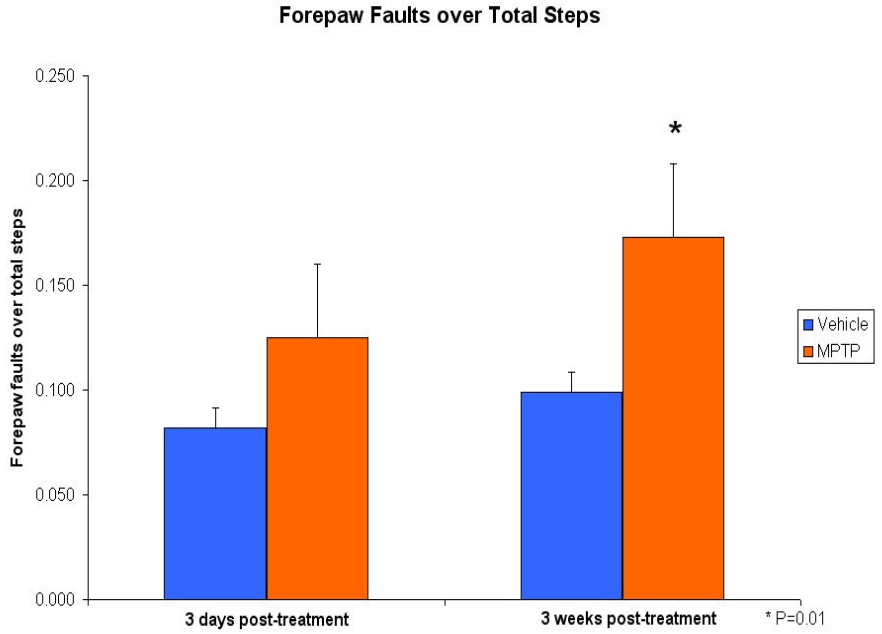


Figure 2. Bar graph showing the ratio of forepaw footfaults on the grid for mice treated chronically with MPTP and euthanized either 3 days post-treatment or 3 weeks later. MPTP-treated mice consistently make more faults on the grid by 3 weeks.

After characterizing the brains of the mice, we began our RNA studies. Over the course of the first year, we examined the splicing pattern of several transcripts that may be associated with neurodegeneration. The results are presented in Table 2. Many of these genes did not show a change in the splice variants after toxin treatment. Four of them did show significant changes with toxin treatment, however, and they are described more fully below.

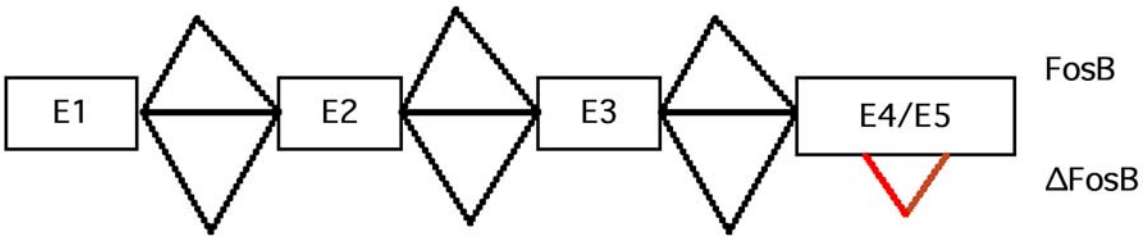
FUNCTION	GENE	BLOOD – ACUTE MODEL	STRIATUM – ACUTE	BLOOD – 3 D CHRONIC MODEL	STRIATUM – 3 D CHRONIC

			MODEL		MODEL
Transcription Factors	FosB	NC	NC	NC	DECREASE
Energy Pathways	Ache	NC	DECREASE	DECREASE	NC
Dopamine Metabolism	Drd2	NS	ND	NS	NC
Signal Transduction	Rgs9	NC	NC	INCREASE	DECREASE
Receptors	GluR2	NS	ND	NS	NC
Cell Cycle	Ania 6A	NC	DECREASE	INCREASE	DECREASE

Legend: NC: no change; ND: not determined; NS: no splice variant detected

Table 2. Analysis of splice variants following acute and chronic MPTP treatment. The ratio of one splice variant to another was compared in total RNA isolated from the blood and striatum of saline and MPTP/probenecid mice following acute and chronic treatment models.

Initially, we examined the changes in splicing of FosB and RGS9 pre-mRNA, since earlier published results indicated abnormal concentrations of proteins produced from the splice variants of these genes were present in the post-mortem brains of Parkinson's disease patients compared to age-matched controls (*18*). Both FosB and RGS9 mediate dopamine signaling in the striatum (*19*) (*20*); (*21*). The gene for the transcription factor FosB produces two mRNAs by intron retention or splicing (Fig. 3). The intron-retained transcript produces full length FosB protein and the intron-spliced transcript produces ΔFosB. ΔFosB is a truncated protein that is missing its carboxyl-terminus and is more stable than FosB (*18*). Our data demonstrate that 3 days after chronic low dose MPTP administration, there is a significant decrease in the ratio of ΔFosB/FosB in the striatum compared to vehicle-treated controls ($P<0.001$), but no change in the ratio of the two transcripts in the midbrain or blood (Fig. 3). The decrease in the ratio of ΔFosB/FosB mRNA did not persist because it was not seen at 3 weeks after treatment (Table 2). No changes in the FosB splice variants were observed in the blood or brains of animals treated acutely with MPTP.



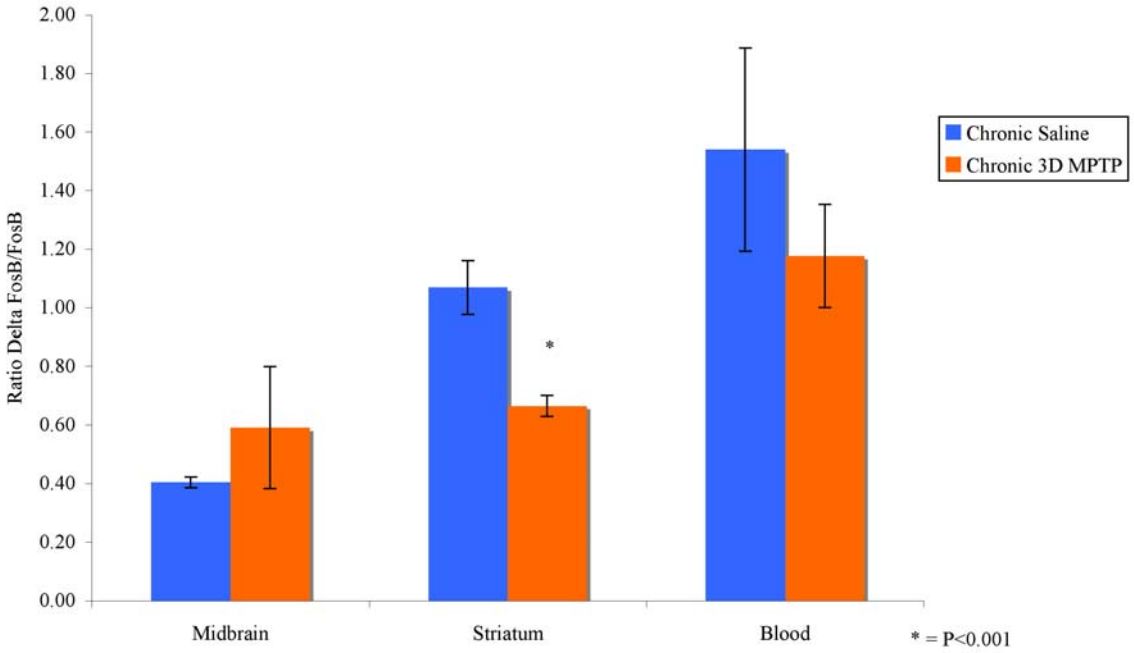
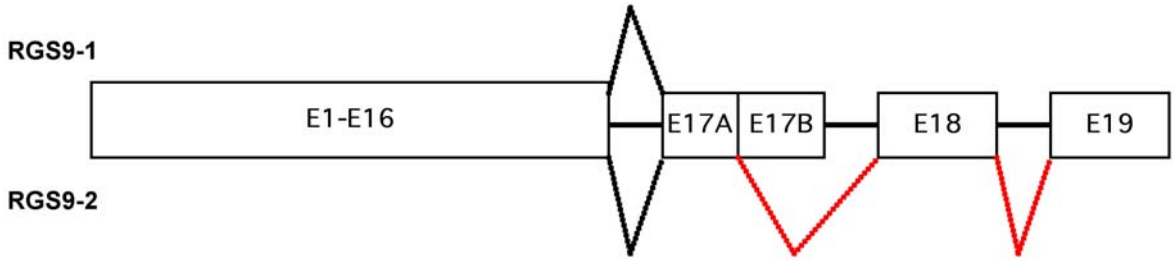


Fig. 3. FosB splice variants in the brain and blood of Parkinsonian mice. The mice were euthanized 3 days (D) after chronic MPTP treatment.

RGS9 is a regulator of G-protein signaling. The RGS9-2 protein is enriched in the striatum, whereas RGS9-1 protein is enriched in the retina (*22*). Both transcripts of RGS9 contain the constitutively spliced exons 1-16 (Fig. 4). RGS9-1 mRNA also contains the entire exon 17 (A and B), whereas RGS9-2 contains exon 17A and exons 18 and 19 due to the use of an internal splice donor site (Fig. 4). Our data demonstrates that 3 days after chronic, low dose MPTP treatment, there is a significant increase in the ratio of RGS9-2/RGS9-1 in the striatum compared to vehicle-treated controls ($P<0.001$). There is no change in the ratio of the two transcripts in the midbrain or blood (Fig. 4). The increase in the ratio of RGS9-2/RGS9-1 mRNA does not persist, because 3 weeks after chronic MPTP treatment, the effect disappears (Table 2). No changes were observed in the blood or brains of the RGS9 splice variants in mice treated acutely with MPTP.



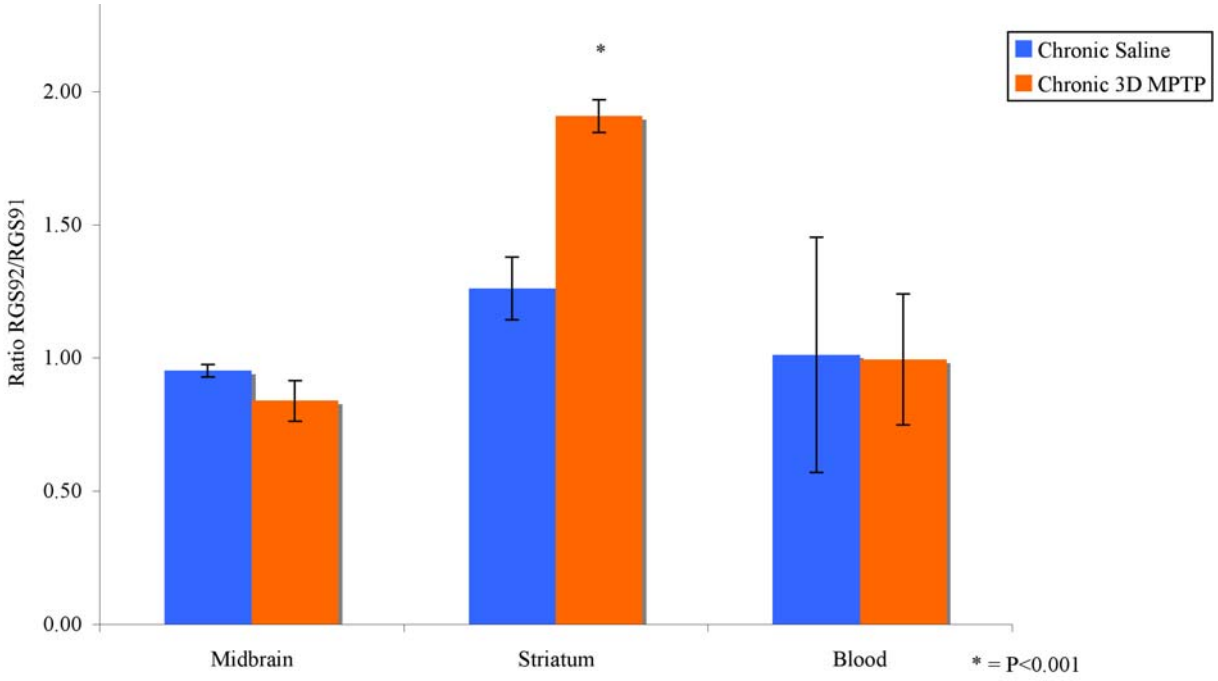


Fig. 4. RGS9 splice variants in the brain and blood of Parkinsonian mice. The mice were euthanized 3 days (D) after chronic MPTP treatment.

To extend the number of biomarkers available for the early detection of Parkinsonism, we also investigated whether the amount of the splice variants of acetylcholinesterase (AChE) changed after MPTP toxin treatment. AChE hydrolyzes acetylcholine (ACh) and thereby functions to terminate Ach-mediated neurotransmission. In many neuropathologies, the characteristics of AChE are modified in the central nervous system. We looked at the AChE-S and AChE-R splice variants. The AChE-S mRNA produces the canonical synaptic tetrameric form of the enzyme. AChE-S is formed by skipping exon 5 such that the final mRNA is composed of exons 1-4 and exon 6 (Fig. 5). The protein produced from this transcript has a cysteine-containing carboxyl-terminus that allows for the formation of the tetramer. The other splice variant we studied, AChE-R, produces a read-through transcript in which a downstream splice donor is spliced to the intron 4 3' splice site such that exon 5 is included in the mRNA (Fig. 5). This produces a protein with a different carboxyl-terminus than the synaptic form. Importantly, the cysteine-containing region of the protein that is needed for tetramer formation is absent. This results in a protein that remains a monomer and thus soluble. We thought AChE-R might be an important splice variant to investigate because it is associated with neurodegeneration and stress-associated disorders (*23*). Our data demonstrate that there is a significant decrease in the ratio of AChE-R/AChE-S in the striatum after acute MPTP treatment compared to vehicle ($P<0.001$), but there is no change in the splice variants of after chronic MPTP administration (Fig. 5). In the blood, there is a significant increase in the ratio of AChE-R/AChE-S after acute MPTP treatment ($P<0.05$, Fig. 5). In addition, there is a significant decrease in the ratio of AChE-R/AChE-S in mice examined at 3 days after chronic MPTP treatment compared to vehicle-treated controls ($P<0.001$). This effect did not persist (Fig. 5). This is a very exciting finding, because it suggests that screening splice variants in the blood of animals exposed to toxins may be useful as an indicator of immediate changes that occur after toxin exposure.

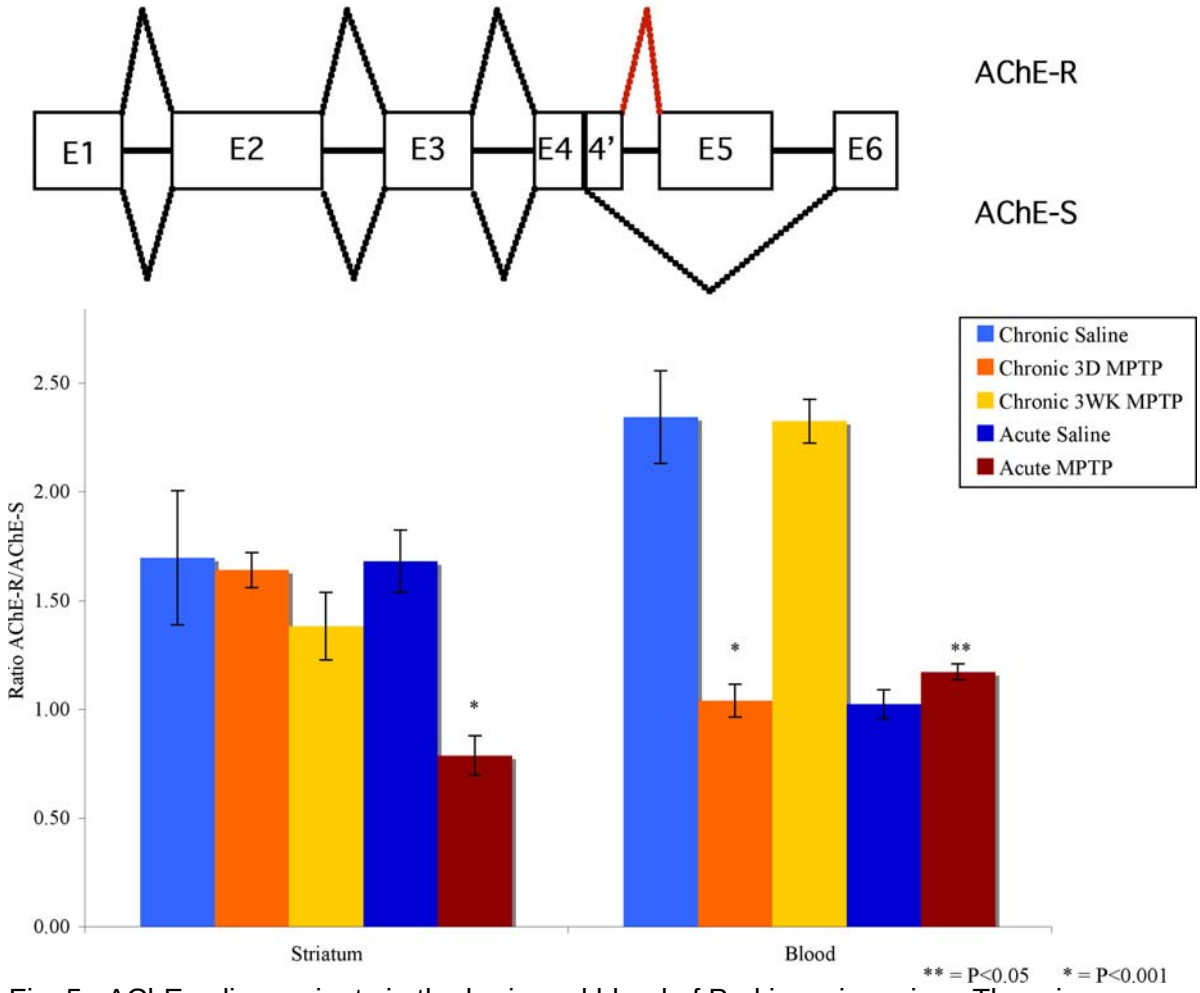


Fig. 5. AChE splice variants in the brain and blood of Parkinsonian mice. The mice were euthanized 3 days after acute MPTP treatment, or 3 days (D) or 3 weeks (WK) after chronic MPTP administration.

We also examined the two splice variants produced from the Ania 6 pre-mRNA. The Ania 6 gene encodes a cyclin, and its expression is induced in the striatum by dopamine stimulation (*24*). The longer splice variant, Ania6, is produced by retention of intron 6', whereas the shorter splice variant, Ania6a, is produced when intron 6' is spliced (*24*). The Ania 6 protein contains a carboxyl-terminus with an arginine/serine-rich domain that is absent from the Ania 6a protein. This region of the protein is important for the association between the Ania 6 protein and splicing factors in the nucleus. To date, no study has examined the Ania6 splice variants in a model for a neurodegenerative disease. This made the choice of these particular splice variants in this study novel. We were therefore particularly excited by the results we have obtained in this part of the study. We observed a significant decrease in the ratio of Ania6a/Ania6 in the striatum of acutely and chronically MPTP-treated mice that were euthanized 3 days post-MPTP/probenecid treatment ($p<0.001$, Fig.6). We also observed an increase in the ratio of Ania6a/Ania6 in the blood of

chronically treated mice that persisted for 3 weeks post-MPTP/probenecid treatment ($p<0.05$, Fig.6). **These results combined with those from the AChE study suggest that using splice variants as biomarkers for neurodegeneration may be beneficial for detecting immediate as well as more persistent changes in the blood after exposure to neurotoxins.**

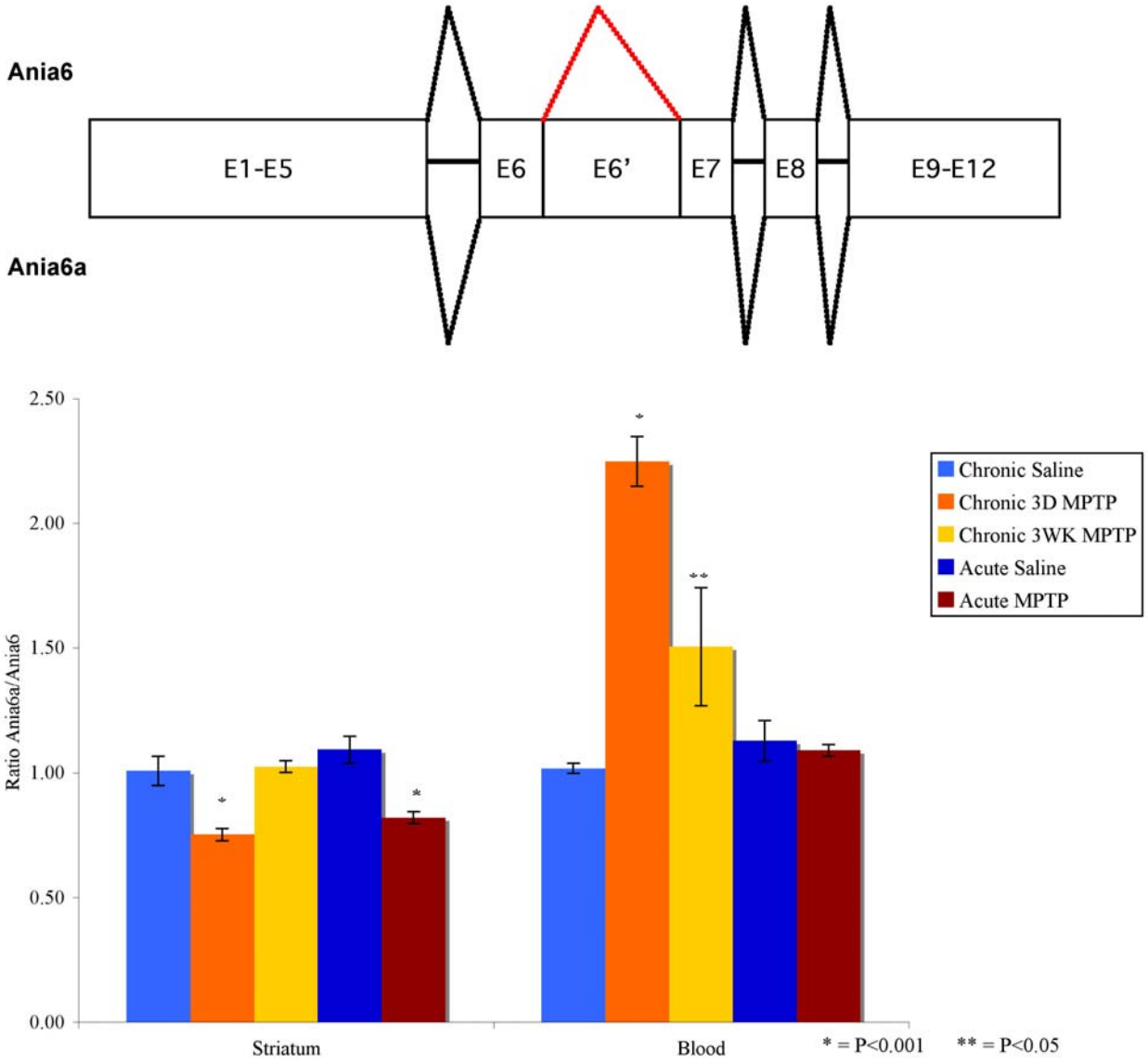


Fig. 6. Ania 6 splice variants in the brain and blood of Parkinsonian mice. The mice were euthanized 3 days after acute MPTP treatment, or 3 days (D) or 3 weeks (WK) after chronic MPTP administration.

Over the course of the next year, we will look at other alternatively spliced transcripts to determine if we can identify additional potential biomarkers for PD. A summary of the genes whose expression may be examined in these studies are shown in Table 3.

FUNCTION	GENE
Apoptosis	Apaf1
	Casp1
	Traf3
Cytoskeletal Proteins	Palm
Oxidative Stress	Oxr1
	NDUFS4
Protein Degradation	Psmc4
	Ube2G1
Receptors	Cd40
Response to Stress	Ercc1
Signal Transduction	Vegf

Table 3. Future Targets. Based upon data gained from microarray gene expression profiling of both substantia nigra pars compacta from Parkinson's Disease patients (*25*) and nigral dopaminergic neuronal cells subjected to oxidative stress (*26*), we have selected a collection of genes with known splice variants. We are currently in the process designing PCR primers for use in examining the splice variants in both our acute and chronic MPTP/Probenecid models.

Manuscripts associated with Objective 1:

Potashkin JA, Meredith GE (2006) The role of oxidative stress in the dysregulation of gene expression and protein metabolism in neurodegenerative disease. *Antioxid Redox Signal*, 8:144-151.

Potashkin JA, Loomis PA, Jodelka F, Jackolin J, Meredith GE (2006) Chronic MPTP administration induces changes in the ratio of splice isoforms of FosB and RGS9 in the striatum of mice. *in prep.*

Objective 2: To determine if the distribution of those splice variants found in rodent models of Parkinsonism correlate with regions in the brain that are affected in PD.

Quantitative *in situ* hybridization histochemistry (ISHH) will be used to determine the distribution of splice variants in the brains of the MPTP-treated mice. In this work, we plan to test the hypothesis that changes in splicing are associated with parts of the basal ganglia where loss of dopamine or its terminals produce Parkinsonism in the animal model. *In situ* hybridization will also allow us to determine if the change in ratio of splice variants correlates with changes in gene expression. We plan to carry out the initial ISHH studies very soon. We will quantify gene expression for splice variants that persist in the brain and blood of the mouse model.

Objective 3: To determine if the splice variants whose regulation is altered in rodent models are altered in the blood of PD patients compared to age-matched controls.

We have recently put together the protocol and consent forms to obtain IRB approval for the human PD studies. Appendix I contains a copy of the protocol submitted for IRB approval and copies of IRB approvals from the University of Chicago and Rosalind Franklin University of Medicine and Science. We are taking advantage of the availability of 2 well-characterized cohorts of PD patients, the first is at the University of Chicago Movement Disorders Center. These patients are registered by the co-Director, Un Kang, M.D. The second cohort is at the Medical College of Wisconsin Movement Disorders Clinic with the director, Karen Blindauer, M.D. IRB permission is still pending at the Milwaukee clinic. Blood will be collected at the time of the patient's initial neurological evaluation. These patients will be mostly drug naïve and in Stage I-II (Hoehn and Yahr scale (*27*)) and will include males and females. We

expect that most patients will be Medicare recipients and, thus, have equal access to health care. Control subjects (n = 25 and age-matched) will be spouses of the patients or obtained from other patient populations. The protocol in Appendix I contains the conditions required for someone to serve as a control. Blood samples will be drawn via venipuncture and collected in heparin-coated tubes. An aliquot (100microl) will be placed in an RNase-free 1.5 ml microcentrifuge tube. All samples will be coded and immediately sent to the Chicago Medical School for RNA analysis.

KEY RESEARCH ACCOMPLISHMENTS:

- Successfully extracted RNA from blood, brain (striatum and substantia nigra) and nasal epithelium of mice treated with MPTP.
- Successfully quantified splice variants for the many genes listed in Table 2 using TaqMan assays (Perkin-Elmer-Applied Biosystems)
- Successfully correlated changes in splice variant ratios with the loss of dopaminergic neurons from the substantia nigra and poor performance on behavioral tests.
- Established that the ΔFos B/FosB ratio decreases in the striatum 3 days after chronic MPTP treatment, but is unchanged at 3 days after acute MPTP procedure (manuscript in prep)
- Established that the RGS9-2/RGS9-1 ratio increases in the striatum 3 days after chronic MPTP treatment, but is unchanged at 3 days after acute MPTP procedure (manuscript in prep)
- Established that that there is a decrease in the ratio of AChE-R/AChE-S in the striatum after acute MPTP treatment compared to vehicle.
- In the blood, there is a significant increase in the ratio of AChE-R/AChE-S after acute MPTP treatment
- Established that there is a decrease in the ratio of Ania6a/Ania6 in the striatum of acutely and chronically MPTP-treated mice 3 days post-MPTP treatment (manuscript in prep)
- Established that there is an increase in the ratio of Ania6a/Ania6 in the blood of chronically treated mice that persisted for 3 weeks post-MPTP treatment (manuscript in prep)
- Obtained IRB permission to study splice variant ratios in the blood of newly diagnosed PD patients. (Appendix 1)

REPORTABLE OUTCOMES

Manuscripts (Appendix 2):

Potashkin JA, Meredith GE (2006) The role of oxidative stress in the dysregulation of gene expression and protein metabolism in neurodegenerative disease. *Antioxid Redox Signal*, 8:144-151.

Potashkin JA, Loomis PA, Jodelka F, Jackolin J, Meredith GE (2006) Chronic MPTP administration induces changes in the ratio of splice isoforms of FosB and RGS9 in the striatum of mice. *in prep.*

Presentations (Abstracts):

Li J, Hong W, Reimers J, Dervan A, Meredith G, Potashkin J. Disruption of splicing regulation in Parkinson¹s disease. RNA2005 Abs 277.

Potashkin J, Loomis P, Pitner J, Leitermann R, Meredith G (2006) FosB splicing regulation is disrupted in the blood of an MPTP rodent model of Parkinson's disease. RNA2006 Abs 666.

Jeitner T, Meredith G, Potashkin J (2006) FosB splicing regulation is disrupted in an MPTP model *Mov Disord 21 (Suppl* 13):P21*.*

Loomis P, Pitner J, Jodelka F, Meredith G, Potashkin JA (2006) Identification of mRNA splice variants in an acute MPTP model of Parkinson's disease. *Soc Neurosci Abs* 822.10.

CONCLUSIONS

The first year of this grant has been fruitful, yielding results in an animal model and taking the first steps to analyze splice variants in human blood. The laboratory personnel work well together and meet often. Progress with identifying splice variants for many different genes has been excellent. Mice treated chronically with MPTP show a shift in the ratio of FosB, RGS9 and Ania6 splice variants in the striatum, 3 days post-treatment. The splicing ratios for AChE and Ania 6 also change in the blood following chronic treatment and, for Ania 6, the changed ratio persists up to 3 weeks after treatment. Progress in the first year includes 4 abstracts, a peer-reviewed publication and an article in preparation. The human studies will begin in the second year.

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APPENDIX 1

TITLE OF PROTOCOL: Identification of Splice Variants as Molecular Markers in Parkinson's Disease

Principal investigator:
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Co-Investigators:
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ASSURANCE VALIDATION: Rosalind Franklin University of Medicine and Science: FWA00005002

CATEGORY OF RESEARCH: Experimental with physiological intervention.

IS THIS RESEARCH SUPPORTED BY A GRANT? Yes. Funding agency: United States Army Medical Research and Materiel Command, NETRP Program. Grant number: W81XWH-05-1-0580

PROPOSED MEDICAL MONITOR: University of Chicago, Dr. Un Jung Kang
PROTOCOL TIMELINE: 3 years (August 1, 2006 – July 31, 2009)

CONTRACTOR AND FACILITY:

As Principal Investigator, I affirm that:
3. The protocol will be carried out in compliance with requirements for the protection of human subjects as provided by Federal and DoD policy.
4. Research conducted under this protocol will conform to the written, approved protocol, including any modifications required by the UC Institutional Review Board.
5. I will personally monitor the progress of this research and the actions of any associate investigators.
6. I will notify the UC Institutional Review Board in writing within 24 hours of any unexpected event or medical misadventure involving research subjects.
7. I will notify the UC Institutional Review Board in a timely manner if either the risk or benefit of the research becomes substantially different than that represented in the protocol.
8. I will provide continuing review reports and a final report for this research as required by UC policy. I will archive the raw data for at least 3 years after study completion.
9. I will transfer all research documents to a UC colleague prior to departing the University, or I will close the protocol.

BACKGROUND AND STUDY RATIONALE:

ABSTRACT

Veterans are exposed through life to toxic environmental agents, substances important in neurodegenerative disease development, especially Parkinson's disease (PD). Current thinking points to the interaction of environmental and genetic factors in PD susceptibility. Alternative splicing, one mechanism that regulates gene expression, is responsible for producing several products from a single transcript and can cause pathogenic changes at RNA and protein levels. A few alternative splice products have been identified in experimental parkinsonism and confirmed in PD patients. Such variants could be used as molecular markers of the disease process, if they could be identified in the blood of individuals at risk.
This proposal will test the hypothesis that the regulation of normal splicing is disrupted in PD, and establish which splice products can be recognized in the blood of PD patients, who are newly diagnosed and not yet on dopamine therapy. Using blood from these patients, alternatively spliced transcripts will be identified, quantified, and the ratios of splice variants for candidate genes, assessed.

PD is a progressive, degenerative disease with no cure. Early detection could improve disease management and enable potential future neuroprotective therapies to be introduced at a stage when they would provide the greatest benefit. The identification of biomarkers in humans using simple molecular assays would significantly advance our ability to identify presymptomatic individuals at risk for developing PD.

RATIONALE: We hypothesize that the abnormal splice variants of transcripts involved in Parkinsonism in experimental animal models will be present in the blood of PD patients and that these variants can be identified and used as a biomarker of the disease process. Peripheral lymphocytes are genetically determined and contain most if not all the same molecules implicated in PD pathogenesis including DA, DA receptors, α-synuclein, parkin, mitochondria, glutathione and glutathione-related enzymes as well as proteasomes (Yoshino et al., 1992; Barroso et al., 1993; Sunada et al., 1998; Barbanti et al., 1999; Caronti et al., 1999; Shin et al., 2000). There is limited evidence to suggest that some of these molecules are different in the peripheral blood lymphocytes of PD patients compared to controls. For example, DA receptors are elevated, whereas blood DA is reduced in PD compared to age-matched controls (Barbanti et al., 1999; Caronti et al., 1999). Mitochondrial enzymes are also changed in lymphocytes of newly diagnosed, untreated PD patients compared to controls (Barroso et al., 1993). RNA can be isolated from both serum and whole blood and we plan to isolate it from both using Promega's Access RT-PCR System.

HYPOTHESIS: Splice variants whose regulation is altered in rodent models are also altered in the blood of PD patients compared to age-matched controls; the regulation of normal splicing is disrupted in PD.

SUMMARY:[1]

HUMAN POPULATION IDENTIFICATION AND BLOOD SAMPLING

[1] Please see Appendix A for Literature cited

We will take advantage of the availability of a well-characterized cohort of PD patients at the University of Chicago Movement Disorders Center, all of whom will be registered by the co-Director, Un Kang, M.D. (see letter of collaboration) and the Medical College of Wisconsin Movement Disorders Clinic, where patients will be registered by the Director, Karen Blindauer, M.D. (see letter of collaboration). The blood will be drawn from 25 PD patients at each of the Movement Disorders Centers at the time of their initial neurological evaluation by Dr. Kang or Dr. Blindauer. These patients will be mostly drug naïve and in Stage I-II (Hoehn and Yahr scale (Hoehn and Yahr, 1967) of PD. We expect that most patients will be Medicare recipients and, thus, have equal access to health care. Although it is important to include Asian and African-Americans in studies such as this, the incidence of PD in these 2 ethnic groups is low compared to that experienced by whites and Hispanics. Therefore, it may not be feasible to include Asian or African-Americans in the study. Control subjects (n = 25 and age-matched) will be obtained from other patient populations at the University of Chicago or Medical College of Wisconsin Clinics, who do not have a diagnosed degenerative disorder. We plan to consider spouses of the PD patients for the healthy age-matched control group. Blood samples will be drawn via venipuncture and collected in heparin-coated tubes. An aliquot (100microl) will be placed in an RNase-free 1.5 ml microcentrifuge tube. All samples will be coded, immediately frozen and sent to the Chicago Medical School for RNA analysis.

TARGET POPULATION AND SAMPLE: Both male and female patients will be recruited. They will be in early stages Parkinson's disease (Hoehn & Yahr stage 1 or 2). They will be eligible if they are between the ages of 40-80 (consider 40-85). This age range is important because we want to recruit individuals during initial stages of the disease and before they are started on dopamine medications. Further, PD is more commonly found to occur in persons in that age range.

SAMPLE SIZE JUSTIFICATION: Our goal is to recruit 50 newly diagnosed patients with Parkinson's disease between both sites[2], and 50 age-matched individuals with no history of neurological disease to serve as controls.

INCLUSION CRITERIA

1. Patient is between the ages of 30 and 85 years (inclusive);
2. Patient has been diagnosed with idiopathic Parkinson's disease based on the presence of a characteristic clinical history and neurological findings including at least 2 of the following: resting tremor, bradykinesia, rigidity, or postural instability;
3. Patient has relatively early disease and has not yet started medications for Parkinson's disease symptoms;
4. Patient scores a 24 or higher on the Mini Mental State Exam;
5. Patient is rated less than 3 on the Hoehn and Yahr PD staging measure;
6. Patient is willing and able to adhere to protocol requirements as evidenced by written, informed consent.

EXCLUSION CRITERIA

1. Patient has clinically significant laboratory abnormalities including renal and hepatic functions elevation greater than twice the upper limit of normal;

[2] University of Chicago and Medical College of Wisconsin

2. Patient has already been started treatment with levodopa/carbidopa alone or with a single, relatively short-acting dopamine agonist, such as pramipexole or ropinirole;
3. Patient has Hoehn and Yahr score of 3 or greater;
4. Patient is taking a prohibited concomitant medication as listed below:
5. The following medications are forbidden for at least one month prior to the study:
    a. Anticoagulants: etomidate, erythromycin, oral azole antifungals, cyclosporine, cisapride, astemizole;
    b. NMDA antagonists: e.g. amantadine, budipine, memantine, remacemide, dextromethorphan;
    c. Any investigational drug;
    d. Drugs which are not used primarily to treat Parkinson's disease but which may modify parkinsonian symptoms: neuroleptics, metoclopramide, compazine, beta blockers;
    e. Drugs with significant muscarinic receptor antagonist activity: Cogentin, Akineton, Artane, Ditropan, Detrol, Elavil, Anafranil, Norpramine, Sinequan, Tofranil, and Pamelor; and including: hyocyamine, enablex (darifenacin), vesicare (solifenacin), sanctura (trospium)
    f. Drugs known to improve dyskinesias: amantadine, dextromethorphan, beta-blockers, fluoxitene, clozapine, quetiapine, olanzapine, buspirone, other anxiolytics, antipsychotics, cannabinoid receptor antagonists, adenosine A2a antagonist; the rationale for excluding f and g is not clear to me. Other putative antidyskinetic drugs could include keppra or topamax.
    g. Drugs known to exacerbate dyskinesias: sodium valproate, CNS stimulants;
    h. Drugs known to have 5HT receptor affinity: ritanserin, sumatriptan and other marketed triptans: zomig, maxalt, frova, etc.
    i. Drugs known to interact with serotonergic mechanisms excluding 5HT3 receptor based antiemetics;
    j. Dopamine agonists known to have a relatively long half-life: cabergoline and pergolide.
6. Patient is pregnant or breastfeeding;
7. Patient has prior bilateral pallidotomy or other ablative surgeries for treatment of PD;
8. Patient has cognitive impairment (MMSE less than 24);
9. Patient has participated in a clinical study with an investigational drug within the last 30 days;
10. Patient has a condition (such as active drug or alcohol abuse) that, in the opinion of the investigators, would interfere with compliance or safety;
11. Patient is unwilling or unable to sign an informed consent and/or to comply with protocol requirements;
12. Patient is known to have one of the inherited, familial forms of Parkinson's disease, as established by a genetic screen.

RECRUITMENT OF SUBJECTS:

Subjects will be recruited at the neurophysiology clinic at the University of Chicago Hospitals Center for Parkinson's Disease and Movement Disorders, and at the Medical College of Wisconsin, Department of Neurology Movement Disorders Clinic.

Subjects will be patients of Dr. Arif Dalvi or Dr. Un Jung Kang or Dr. Karen Blindauer and will be seen in the clinic for a routine clinic visit. If the doctors feel that the patient may meet criteria to participate in the study, they will immediately contact the study coordinator, who will meet with the doctor and patient, to begin the informed consent process.

SUBJECT IDENTIFICATION: study subjects will be asked to provide a blood sample, which will begotten via venipuncture by a trained phlebotomist on the research team. Both the subject themselves and the specimen they provide will be assigned an ID number.[3] Names of subjects and any other identifying information (personal health information, or PHI) will be known and available only to the research personnel at the University of Chicago and Medical College of Wisconsin.

INFORMED CONSENT PROCESS:

Note: All patients must be able to provide consent to participate. Consent by a legally authorized representative is not acceptable.

The study coordinator will provide a copy of the consent form to the patient and will go through it with them in full detail, giving the patient time to ask any questions he/she may have regarding the study. Once the consent form has been signed by the patient, the study coordinator, and the doctor, the latter two will go through the inclusion and exclusion criteria together and will determine whether or not the patient meets all criteria for participation. If the subject does not meet criteria, he or she will be informed and will not be asked to provide a blood sample, or to continue in the study.

SAMPLING METHOD: subjects will be obtained using convenience as they report to the clinic or if time does not allow, they may return on a separate day to complete study related activities..

COLLECTION OF SAMPLES

The duration of the study is anticipated to be 3 years. **A total number of 50 males and non-pregnant females recently diagnosed with Parkinson's disease will be included in the study.** Individuals similar in age with no history of Parkinson's disease will serve as controls. The study involves no more than minimal risk to the subjects. There is no need for extra blood drawn from the subjects. Blood specimen (2.5 ml) will be drawn in PAXgene Blood RNA tubes (Qiagen) by a registered nurse. The samples will be stored for up to 5 days at 2-8^{0}C prior to further processing. The study will be done in collaboration with the physicians who are treating Parkinson's patients. The study subjects and controls will be recruited from the University of Chicago and Medical College of Wisconsin. The scientific rationale for the research is to evaluate RNA biomarkers for the detection of Parkinson's disease in patients prior to drug treatment. Study subjects and controls will be asked to participate in the study during the initial visit in the clinic.

DATA COLLECTION[4]

SCREENING PROCEDURES:

Screening procedures for this study will involve pre-screening involving medical record review on current patients of Dr. Kang and Dr. Dalvi at the University of Chicago Hospitals, and Dr. Blindauer at the Medical College of Wisconsin to confirm diagnosis of Parkinson's Disease and

[3] Please see Appendix C

[4] Please see Appendix B for study schematic outline

review of medical as well as medication/treatment history. If the patient has already been seen by Dr. Kang or Dr. Dalvi in the past, and meets criteria as determined by Dr. Kang and Dr. Dalvi and the study coordinator, he or she will be considered eligible to participate in the study and will be taken through the informed consent process. If the patient is a new patient of Dr. Kang, Dr. Dalvi's or Dr. Blindauer's, he or she must undergo a clinical evaluation typical of such normative neurology clinic visits. At said visit, if Dr. Kang, Dr. Dalvi or Dr. Blindauer feels the patient may be a good candidate for the study, the patient will then be briefed of the study, and if interested in participating, will be taken through the informed consent process at this same time or, if time does not permit, with return to the center to complete study related activities at a separate time. Once the new patient is consented and is now a study subject, the doctors and study coordinator will review the patient's medical history including past/current medications/treatments to ensure they are entirely compliant with exclusion/inclusion criteria. It is imperative to be able to thoroughly document the patient/study subject's treatment history for Parkinsons' disease. Therefore, this history will be thoroughly screened for and evaluated prior to collecting the blood for this study. If the patient/study subject's history shows that he/she does not in fact meet study criteria, he or she will not be asked to participate, or to provide a blood sample. If a blood sample has already been taken and it is discovered through further evaluation of the patient/study subject's history or present medical condition, that he/she does not meet criteria to participate, (for example, if he/she scores $\leq$ 24 on the MMSE), the blood sample that has already been taken from said person will not be included in the study data set, and the specimen will be destroyed.

LABORATORY EVALUATIONS:

COLLECTION OF SAMPLES: Blood specimen (2.5 ml) will be drawn in PAXgene Blood RNA tubes (Qiagen) by a member of the research team; each sample will be coded with an ID number. Samples will be stored for up to 5 days at 2-8^{0}C prior to further processing.

EVALUATIONS TO BE MADE: RNA will be prepared from the blood using Tri-Reagent BD (Molecular Research Center, Inc.). The extracted RNA will be amplified by RT-PCR. Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) will be used for first strand DNA synthesis and the *Thermus flavus* DNA polymerase used for the second synthesis and further amplification. We use a two-reaction/one test tube method in order to eliminate contamination problems that arise when a separate test tube is required for each step. The reverse transcription is incubated at 48^{0}C for 45 min. To maximize the sensitivity of the assay, the magnesium sulfate concentration will be optimized (varied from 1- 3 mM) for the each primer combination. After the room temperature incubation, the reaction is incubated at 94^{0}C for 2 min. to inactivate the AMV reverse transcriptase and denature RNA/cDNA hybrids. Inactivation of the reverse transcriptase allows for high yields during amplification.

Using Real time RT-PCR, the reverse transcription and amplification steps are carried out in a thermocycler/detector connected to a computer so results are appraised in real time. Fluorescent reporters, such as SYBR green, will be used to detect reaction progress during each amplification cycle. This will allow extension of a reaction for further amplification when a low abundant transcript is being analyzed, or terminate a reaction when it is complete. The temperature of the annealing and extension reactions will depend on the individual primer combination. Usually the annealing temperature is from 42-60^{0}C and thee extension temperature from 62-68^{0}C. The higher the melting temperature of the primers, the higher the temperature will be for both

incubations. Using high temperatures will increase the specificity of the PCR products. Usually 35 to 40 cycles of amplification are sufficient; 50 cycles being required for rare RNAs. A final 7 min extension at 68^0C will be included to assure that the final product is fully extended.

An amplification plot for each sample will be generated showing the increase in reporter dye fluorescence with each cycle of PCR. From the plot, a threshold cycle (Ct) value, which represents the PCR cycle number that produced fluorescence detectable above an arbitrary threshold, is calculated. The amount of template at the start of the reaction determines the Ct. The more abundant the target transcript at the start, the fewer cycles are needed to detect fluorescence above threshold. For these experiments, relative standard curves will be sufficient to compare one splice variant to another from the same gene.

STORAGE: The blood samples will be stored for up to 5 days at 2-8^0C prior to further processing.

LABS PERFORMING EVALUATIONS AND SPECIAL PRECAUTIONS:

- The Laboratory of Dr. Judy Potashkin at the Rosalind Franklin University of Medicine and Science will perform the laboratory evaluations.
- The collection of blood presents minimal risks to the study subjects. These risks may include: bruising, swelling, infection or excessive bleeding in area of venipuncture. The member of the research associate who performs phlebotomy do so in the clinically appropriate way, allowing for the safest means possible for this to occur, and to minimize the chances of bruising, swelling, infection, or excessive bleeding. There is also the rare possibility of vasovagal syncope occurring after the venipuncture. Subjects experiencing lightheadedness during or after the blood draw will be asked to lie supine until symptoms subside to prevent a syncopal episode from occurring. IF an adverse event occurs, the research team physician will be contacted and the IRB notified immediately. Any adverse events that occur will be documented and reported according to the guidelines set forth by the US Federal Government, NIH and FDA.
- The research associate will take standard safety precautions to avoid the risk of infection including wearing a lab coat, disposable gloves and protective goggles.
- The RNA solutions and blood mixture for the PAXgene Blood RNA tube will be disinfected using 1 volume of bleach (5% sodium hypochlorite) per 9 vol of RNA stabilizing solution and blood mixture. Sample preparation waste will be autoclaved to destroy any possible infectious material.

CLINICAL ASSESSMENTS:

Clinical assessments will involve the routine exam performed by the neurologist including but not limited to: physical and neurological exam, Unified Parkinson's Disease Rating Scale, vital signs; the patient will discuss their medical and treatment history, including past and present medications, with the doctor and the study coordinator. The study coordinator or investigator will administer the Mini Mental State Examination (MMSE); if the patient scores <24 on the MMSE, he or she will not have met all criteria for participation, and will not be included in the study. The patient's medical records will be reviewed to gain a clear picture of their medical history so their eligibility can be confirmed. A Hoehn and Yahr will be completed in order to stage the severity of the patient's PD

METHODS USED FOR DATA COLLECTION: We will use a set form (see attached) for recording data.

Data will be kept as hard copy in a laboratory notebook and as an electronic copy on a dedicated computer.
Blood samples will be taken at the University of Chicago and documented. Samples will be shipped to the Chicago Medical School, Rosalind Franklin University of Medicine and Science

DATA MANAGEMENT
DATA ANALYSIS: Data will be collected on paper, and archived electronically in Dr. Potashkin's laboratory. The data will be password protected.

DISPOSITION OF DATA: Data will be maintained electronically under Dr. Potashkin and Dr. Meredith for 3 years. Hard copies of the data will be kept for 10 years, also with Drs. Potashkin and Meredith. During all 10 years, the data will be available only to Dr. Meredith, the co-investigators, and members of the research team personnel.

CONFIDENTIALITY: Research subjects will be protected by an ID code issued at the time of their visit. That code and associated data will be held at the University of Chicago and Medical College of Wisconsin by the research coordinator. All patients and their PHI will be protected in accordance with HIPAA regulations.

RISKS/BENEFITS ASSESSMENT

- Risks: There is a minimal risk of the following due to venipuncture: bruising, inflammation, infection, syncope or excessive bleeding. There is also a minimal risk of loss of privacy; however, all precautions will be taken to ensure the complete protection of the patient's privacy.

- Precautions: The laboratory personal will take standard safety precautions to avoid the risk of infection including wearing a lab coat, disposable gloves and protective goggles. The risks to the research subjects related to venipuncture will be minimized using a sterile technique, compression bandage when needed for bleeding at the puncture site, and having the subject lie supine to prevent syncope if vasovagal symptoms occur.

- Special Care Needs: All patients will be provided with the best care possible in the clinic setting, and in accordance with the GCP guidelines. All laboratory work will be completed professionally and according to the current guidelines for laboratory professionals.

- Benefits: There is no direct benefit to the patient to participate in this research study. There may be an indirect benefit in that patient will be a part of an important study which could lead to improved understanding of PD, and subsequently to potential future treatments of PD, and greater understanding of neurodegenerative disease.

EVALUATIONS TO BE MADE:
DATA TESTING AND ANALYSIS
RNA will be prepared from blood using Tri-Reagent BD (Molecular Research Center, Inc.). The extracted RNA will be amplified by RT-PCR.

An amplification plot for each sample will be generated showing the increase in reporter dye fluorescence with each cycle of PCR. From the plot, a threshold cycle (Ct) value, which represents the PCR cycle number that produced fluorescence detectable above an arbitrary threshold, is calculated. The amount of template at the start of the reaction determines the Ct. The more abundant the target transcript at the start, the fewer cycles are needed to detect fluorescence above threshold. For these experiments, relative standard curves will be sufficient to compare one splice variant to another from the same gene.

LABS PERFORMING EVALUATIONS AND SPECIAL PRECAUTIONS

The laboratory personal will take standard safety precautions when handling the blood specimens to avoid the risk of infection including wearing a lab coat, disposable gloves and protective goggles.

The RNA solutions and blood mixture for the PAXgene Blood RNA tube will be disinfected using 1 volume of bleach (5% sodium hypochlorite_ per 9 vol of RNA stabilizing solution and blood mixture. Sample preparation waste will be autoclaved to destroy any possible infectious material.

MODIFICATION OF THE PROTOCOL

Any changes to be made to the protocol in the time following first IRB approval will be made in the form of an amendment and no procedures or evaluations will be completed outside of what is written and approved of in this protocol without the approval of the IRB via amendment.

APPENDICES

A. LITERATURE REFERENCES

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B. SCHEMA

Evaluations	Clinic/ Study Visit 1
Medical Records Review5	X
Neurological Exam	X
Physical Exam	X
Vital Signs	X
Informed Consent	X
MMSE6	X
Blood draw	X
Shipment of specimens	X
New UPDRS	X

C.IDENTIFICATION NUMBERS AND LABELS:

Numbers will be assigned in the following *alpha-numeric* fashion:

UC =University of Chicago
+ 3 digit number 001, 002, 003, in order of enrollment into study.
e.g.: UC-001, UC-002, …

WI= Medical College of Wisconson
+ 3 digit number 101, 102, 103, in order of enrollment into study.
e.g.: WI-101, WI-102, …

Labels will be used to head all data forms and on all collection tubes in the following format:

ID: _____Date:_____

DOB: _____ Sex: M / F

[5] Medical record review may be completed prior to clinic/study visit if patient is already a patient of the participating physician.
[6] MMSE=mini mental state exam

APPENDIX 2

JUDITH A. POTASHKIN and GLORIA E. MEREDITH

The Role of Oxidative Stress in the Dysregulation of Gene Expression and Protein Metabolism in Neurodegenerative Disease

ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 1 & 2, 2006

Forum Review

The Role of Oxidative Stress in the Dysregulation of Gene Expression and Protein Metabolism in Neurodegenerative Disease

JUDITH A. POTASHKIN and GLORIA E. MEREDITH

ABSTRACT

There are few examples for which the genetic basis for neurodegenerative disease has been identified. For the majority of these disorders, the key to their understanding lies in knowledge of the molecular changes that contribute to altered gene expression and the translational modification of the protein products. Environmental factors play a role in the development and chronicity of neurodegenerative disorders. Environmental stimuli such as hypoxia, toxins, or heavy metals, increase production of reactive oxygen species and lower energy reserves. Chronic exposure to oxidative radicals can adversely affect gene expression and proteolysis. This review summarizes what is currently known about some of the changes in gene expression and protein metabolism that occur after oxidative stress which contribute to neurodegeneration, and reveals areas where more research is clearly needed. *Antioxid. Redox Signal.* 8, 144–151.

INTRODUCTION

OXIDATIVE STRESS occurs when the production of reactive oxygen species (ROS), a normal product of cellular metabolism, is greater than the ability of the cell to repair the resulting damage. In neurodegenerative disease, susceptibility to oxidative stress may be genetically influenced but environmentally activated, (i.e., by exposure to toxins, heavy metals, viruses or hypoxia). This review will explore the oxidative mechanisms underlying the dysregulation of gene expression and damage to protein metabolism. These data should be useful in gaining insight into the molecular mechanisms that underlie cell death in neurodegenerative disease.

OXIDATIVE STRESS AND NUCLEIC ACID DAMAGE

Free radical-mediated tissue damage may be caused by endogenous processes such as an inflammatory response or by exogenous irritants. Water, the most abundant molecule in our body, is very sensitive to these processes. Normally water is a stable molecule, but energy bursts from heat or radiation may split one of the shared electron bonds, producing unpaired electrons on a hydrogen ion and a hydroxyl radical. In an attempt to reconstitute water, free radicals try to pair with other hydrogen atoms and in so doing exert oxidant stress on substrates in the vicinity. Nuclear DNA is often not affected by oxidative stress because the nucleus is poorly oxygenated and the DNA is bound by histones that quench radicals. Mitochondrial DNA, however, is very sensitive to oxidative damage because of its proximity to the respiratory chain, absence of protective histones, and limited DNA repair capabilities (57).

One common product of nucleic acid damage by oxidation is 8-hydroxyguanosine (8OHG). Indeed, 8OHG immunoreactivity is widely used as a marker for evaluating the effect of oxidative stress on nucleic acids. This molecule can be induced by various environmental factors and is known to permanently damage cytoplasmic RNA and mitochondrial DNA, thereby contributing to neurodegeneration. For example, in Parkinson's

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disease, there is increased 8OHG immunoreactivity in neurons of the substantia nigra (78). Patients with multiple system atrophy (MSA) and dementia with Lewy bodies (DLB) also show increased 8OHG immunoreactivity in substantia nigra neurons compared to controls, but less than in Parkinson's disease patients (78). In a separate study, an increase in 8OHG was an early event in Alzheimer's disease (50). In this study, oxidative damage decreased with disease progression suggesting that a compensatory change reduced the damage. Even though studies such as this suggest that oxidative stress is a primary cause of neurodegeneration, it remains unclear whether it is a cause or consequence (4). Certainly, neuronal vulnerability to free radical stress is dependent upon a number of factors, either environmental, genetic or both. For example, offspring of maternal victims of PD have pronounced deficits in Complex I that are responsible for increasing ROS (64).

DNA damage cannot always be repaired properly, a situation that can precipitate cell death. An example of this is Cockayne syndrome (CS), which is caused by mutations in the CSA, CSB, or the xeroderma pigmentosum genes (e.g., XPG). The transcription-coupled repair pathway that involves the XPG and CSB proteins repairs 8OHG lesions, and defects in this pathway are the cause of this syndrome (13, 39, 71). Another example of DNA damage and how the lack of repair induces cell death is found in amyotrophic lateral sclerosis (34, 67). Damaged DNA accumulates in familial amyotrophic lateral sclerosis and 8OHG lesions increase in the motor cortex of patients with the sporadic form of the disease (9, 20). Since the accumulation of DNA damage is a signal for apoptosis, such damage may be partially responsible for the death of upper and lower motor neurons in amyotrophic lateral sclerosis. Additional mechanisms of neurodegeneration, such as mitochondrial dysfunction leading to an increase in free radical generation, may also be involved in sporadic forms of the disease (20).

Cytoplasmic RNA is also vulnerable to oxidative damage. In Alzheimer's disease, selective classes of mRNA species are particularly susceptible, and many of the damaged mRNAs encode products that have been implicated in disease pathogenesis (61). When the damaged mRNAs are expressed in cell lines, they are improperly translated, which leads to protein aggregation (61).

Together these studies suggest that modification of nucleic acids by oxidative stress could be an important feature of neurodegenerative disease. In Cockayne syndrome, correction of the defect using gene therapy might be one therapeutic treatment. In Parkinson's and Alzheimer's disease and in amyotrophic lateral sclerosis, which have 8OHG damage, stimulating DNA repair through antioxidants may be beneficial (11).

Even though the evidence is strong that oxidative stress is damaging, limited production of ROS may be benign or even beneficial. A number of studies have proposed that the generation of ROS is required for energy supplies' signals to reach the nucleus from the mitochondria. Under pathological conditions, ROS increase, which may affect not only electron transport within the mitochondria but also the number and permeability of mitochondria themselves. Under a certain level, ROS presumably induce stress responses by altering the expression of specific nuclear genes to uphold energy metabolism in order to rescue the cell. Once beyond a certain threshold, free radicals can damage mitochondrial and nuclear DNA and induce apoptosis by increasing mitochondrial membrane permeability (38).

OXIDATIVE STRESS AND RNA METABOLISM

Gene expression analysis in neurodegenerative diseases

Several studies have used microarray analysis to examine the effect of oxidative stress on gene expression in various models of neurodegenerative disease. Oxidative radicals have been shown to disrupt the regulation of expression of several genes in the nigral dopaminergic cell line SN4741, including those that encode subunits of Complex I, exocytosis and membrane trafficking proteins, oxidoreductases, and regulatory molecules of apoptosis (75). Several of the genes identified in this study have previously been implicated in Parkinson's disease. They include the B8 and B17 subunits of mitochondrial complex I, which were down regulated, and syntaxin 8 and heme oxygenase-1, which were up regulated (75).

In chronic MPTP- and 6-hydroxydopamine-induced parkinsonian rodent models, microarray analyses show that the expression of genes associated with oxidative stress, inflammation, glutamate and neurotrophin pathways, cytoskeleton, cell cycle control, apoptosis, and signal transduction pathways are significantly altered in toxin-treated animals compared to controls (26, 27, 42, 47). When combined, these studies suggest that a dysregulation of gene expression in the substantia nigra of Parkinson's disease models could eventually lead to dopaminergic neuronal death.

An alternative approach to studying gene expression is to use a candidate gene method. Reverse transcriptase-polymerase chain reaction was used by Aksenov et al. (3) to study changes in the expression of key oxidative stress handling genes in Alzheimer's disease. The results indicated that Mn-SOD mRNA normalized to β-actin was unchanged, but Cu/Zn-SOD mRNA was increased in Alzheimer's disease patients compared to controls. These investigators also noted a general decrease in transcription in Alzheimer's disease brains. Together these data suggest that region-specific changes in ROS-mediated injury rather than a decrease in oxidative stress handling genes, contribute to the neurodegeneration observed in Alzheimer's disease brains (3).

From these examples it is clear that microarray studies benefit the study of neurodegenerative disease by characterizing affected genes and identifying molecular probes that may be used as biomarkers for monitoring neurodegeneration or the benefits of drug therapy. To use the candidate gene methods, one must have some prior information about the genetic basis of the disease, which unfortunately limits their utility. Complete identification of gene expression signatures after exposure to oxidative stress, would certainly help in identifying factors important in disease development and progression.

Transcriptional dysregulation

Modification of transcription factors by oxidation or alkylation can affect both protein–protein and protein–DNA

interactions and thereby alter their activity. In particular, many transcription factors have zinc finger domains that require two or four zinc-coordinated cysteine sulfydryl groups that must be in the reduced form. When oxidative stress induces cysteine oxidation within the zinc finger domain, the zinc coordination is lost and the secondary structure is distorted (73). Among zinc finger transcription factors that are induced by persistent oxidative stress, is Sp1. *In vivo*, the hyperoxidative repression of Sp1 transcription from promoters with essential Sp1 binding sites, includes the simian virus 40 early region, glycolytic enzyme, and dihydrofolate reductase genes (74). Zinc finger transcription factors most likely act as redox sensors and thereby mediate some of the transcriptional changes that occur (reviewed in 73). The quantity and availability of intracellular zinc are important factors for determining the oxidative state of zinc finger transcription factors (73).

An interesting twist to the story of how modified transcription factors may lead to neurodegeneration is that of myocyte enhancer factor 2 (MEF2), a transcription factor that plays a role in neuronal survival (43). A recent study showed that oxidative stress induced phosphorylation of MEF2 by Cdk5 kinase, which inhibits the activity of this factor (23). Since MEF2 is required for survival gene expression, the end result of its phosphorylation is neuronal apoptosis. Previous to this work, the deregulation of Cdk5 activity was shown to be important for pathogenesis of Alzheimer's disease and amyotrophic lateral sclerosis (48, 55, 76).

The induction or repression, in expression of various transcription factors, is also likely to play a role in neurodegeneration. For example, oxidative radicals may stimulate protein kinase cell signaling pathways (32, 63). This activation leads to increases in the transcription of some genes including the transcription factors NF-kB, AP-1 and Sp1, which have all been implicated in redox-modulated gene expression (1, 24, reviewed in 16). Metals such as aluminum, zinc, and lead can also trigger oxidant-sensitive transcription factors (reviewed in 54). Aluminum and lead induce oxidative stress by interacting with ROS, affecting membrane rheology and signaling cascades (54). Aluminum has been associated with the etiology of amyotrophic lateral sclerosis, and in Parkinson's and Alzheimer's diseases. Zinc also increases oxidative stress and affects the activity of redox-sensitive transcription factors Erg-1, AP-1, and NF-κB (41, 53, reviewed in 37, 60, 63).

Dysregulation of pre-mRNA processing

The effect of oxidative stress on pre-mRNA editing and splicing has yet to be thoroughly investigated. There are several studies, however, that suggest that regulation of these RNA processing events is disrupted by oxidative stress. Examples include the editing of the GluR2 subunit of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in amyotrophic lateral sclerosis, and in Alzheimer's and Huntington's diseases (2, 65). A glutamine of GluR2 is changed to an arginine residue, thereby altering the calcium conductance of the receptor. In amyotrophic lateral sclerosis, this editing event is reduced substantially in the ventral gray area of the spinal cord, and could be responsible for increased calcium influx, and perhaps, motor neuronal death (65). A similar change in editing was observed in the prefrontal cortex in Alzheimer's disease and in the striatum in Huntington's disease (2).

One study that looked at changes that occur in an ischemic brain indicated that several splicing regulatory factors were translocated from the nucleus to the cytoplasm (14). The result of this redistribution after oxygen deprivation was a change in alternative splice site selection of the interleukin-1β converting enzyme homolog 1, an enzyme that functions in apoptosis. In another study, hypoxia induced a change in alternative splicing of the presenilin 2 gene in which exon 5 was skipped (58). This is the same change in splicing regulation seen in Alzheimer's disease brains. In addition to these examples, there are numerous other neurodegenerative diseases that show changes in the regulation of alternative splicing, which lead to a shift in the normal ratio of one splice variant to another (reviewed in 17, 19, 22, 25).

OXIDATIVE STRESS AND PROTEIN METABOLISM

In the past decade, intracellular aggregation of proteins has become increasingly recognized as an important pathological hallmark of neurodegenerative disease. Such aggregations appear in numerous disorders including Parkinson's disease, DLB, MSA, and in Huntington's and Alzheimer's diseases, and are characterized by common pathological features. The aggregates contain misfolded or post-translationally modified proteins, are primarily located intracellularly in the cytoplasm of neurons or glia, and are associated with high levels of ROS (31, 40). In Huntington's and Alzheimer's diseases, the respective intracellular aggregates of huntingtin and tau are toxic (15, 35). However, recent work by Arrasate *et al.* (5) questions that toxicity. They, like others (8, 62, 66, 70), have raised the possibility that the isolation of toxic proteins into inclusions are a neuron's normal response to the accumulation of abnormal proteins. In Parkinson's disease, the intracellular inclusions are called Lewy bodies and contain α-synuclein. This protein, which also accumulates with huntingtin in Huntington's disease (15), and serves as the precursor of the nonamyloid component of plaques (NACP) in Alzheimer's disease (29), may be toxic when it is in the form of small aggregates called protofibrils (72). Nevertheless, their accumulation in Lewy bodies may not be toxic. Since mature Lewy bodies are found in surviving neurons in Parkinson's disease, they may reflect a cell's defense to the presence of toxic molecules (5, 66). The intermediate steps to inclusion formation, however, may not be so 'cell'-friendly and could increase a neuron's exposure to pathogenic proteins (see below).

Human α-synuclein is a 140 amino acid molecule that has a highly hydrophobic domain involved in amyloid formation (29). This protein has been detected in Lewy bodies in Parkinson's disease and DLB and in aggregates in glia cells in MSA (31). Aggregated α-synuclein generally forms insoluble β sheets (10), but how this natively unfolded molecule is transformed remains unresolved. Nevertheless, some clues can be found in how oxidative stress affects the translation of the protein, as noted earlier, or modifies it post-translationally (51, 56).

Two mouse models induced by toxin exposure—chronic MPTP/probenecid and rotenone—have α-synuclein- and ubiquitin-immunoreactive aggregates in the cytoplasm but not in the nuclei of nigral dopaminergic neurons (7, 46). Various *in vitro* models also show intracellular aggregation of these proteins, especially in the presence of human α-synuclein or iron (12, 52). In all these cases, the aggregations are not organized as Lewy bodies, but rather as small granular and fibrillar structures that are referred to as "Lewy-like" inclusions (45). Nevertheless, the aggregation process may mimic that in Parkinson's disease including the dependence on enhanced ROS production due to mitochondrial damage (reviewed in 40).

Misfolded, unassembled, or damaged proteins are generally degraded in an ubiquitin-dependent manner by a nonlysosomal, ATP-dependent, protein degradation pathway or ubiquitin-proteasomal system (UPS) (6). This pathway may become dysfunctional or overloaded if the cell has a reduced capacity for ATP production, as occurs under conditions of prolonged oxidative stress (45). Since ubiquitin appears to be an important chaperone of α-synuclein, establishing a link between UPS malfunction and increased ubiquitinated complexes in Lewy bodies may shed light on how proteolysis changes in Parkinson's disease (21).

Post-translational modifications of proteins are part of the degenerative process in Alzheimer's, Huntington's and Parkinson's diseases, and may play a role in inclusion formation. In Parkinson's disease, we know that α-synuclein can be nitrated, hyperphosphorylated, or phosphorylated at tyrosine residues; enhanced oxidative stress increases the rate at which tyrosine is modified (56, 59). Such alterations will alter the hydrophobicity and conformation of the protein, thereby enabling polymer aggregation (56). Abnormal protein phosphorylation may also modify the ability of a protein to bind lipids and affect triglyceride turnover (18).

There is good evidence that lysosomes are capable of carrying out limited proteolysis, especially if the UPS is malfunctioning. However, these organelles also contain the major pool of redox-active labile iron within the cell, and in the presence of oxidative radicals, this iron can relocate to the nucleus where it can damage DNA (36). Interestingly, recent studies provide convincing evidence for a link between proteasomal inhibition and lysosomal proteolysis. Impairments in the UPS system increasingly shunt proteins to lysosomal proteolysis. However, elevations in protein oxidation, nitration, or phosphorylation slows protein turnover and can thereby enhance the aggregation of proteins (33). Lipofuscin granules, which are formed in lysosomes by an iron-catalyzed oxidation of protein and/or lipid residues, further sensitize lysosomes to oxidative stress (69). Oxidative radicals, such as hydrogen peroxide, diffuse easily into the granules where they reduce ferrous iron and form peroxidation byproducts, such as protein carbonyls (45). The low pH in the lysosomal compartments produces an environment compatible with these activities. Lipofuscin granules accumulate with age. However, the progressive accretion of indigestible residues, as demonstrated in Alzheimer's and Parkinson's diseases (8, 49), accelerates lipofuscin formation (69). Lipofuscins can interfere with the autophagic process by which most lysosomal contents are normally degraded, suggesting that these granules reflect lysosomal dysfunction and, presumably, a decline in protein degradation (69). Although the increase in these granules is part of the normal aging process, their appearance is dramatically accelerated in various neurodegenerative diseases (44, 49) as well as in dopaminergic neurons in Parkinson's disease (45). Lipofuscin granules are also numerous in brain stem neurons outside of the substantia nigra in Parkinson's disease (8).

In Parkinson's disease, dopaminergic neurons, lipofuscin granules are intimately associated with abundant neuromelanin. Neuromelanin, normally synthesized enzymatically in lysosomes, co-localizes with a large accumulation of iron (77). Although reduction in neuromelanin content is reported in Parkinson's disease, this may be due to the loss of neuromelanin-containing dopaminergic neurons and not to decreased levels in lysosomes of remaining neurons. The cellular content of lipofuscin and neuromelanin may be important for Lewy body formation, since lipofuscin substructure is granular and associated with lipid as early stage Lewy bodies show (30, 45). The high lipid content of lysosomes and Lewy bodies may also play a role in protein accretion and serve as an important source of hydrogen bonds for protein nitration as well as contribute to an increase in the length of protein filaments (21, 28). Overloaded lysosomes with their lipofuscin, iron and lipid contents, may rupture, as they have been shown to do under *in vitro* conditions (79). The contents would then be expelled into the cytoplasm where they would further damage mitochondria and its DNA. Moreover, lipofuscins could form nucleation centers for protein filaments as a first step in the process of Lewy body formation (45).

SUMMARY AND CONCLUSIONS

Significant molecular changes can occur in neurodegenerative disease at every level of gene expression from transcription through post-translation modification of proteins, and each may play an important role in neuronal death. Figure 1 summarizes the prominent pre- and post-translational modifications that are associated with prolonged oxidative stress and lead to neuronal injury and death. Mitochondrial DNA, which is particularly sensitive to oxidative injury (57), contributes significantly to reduced levels of ATP and increased production of ROS when damaged. The incorporation of 8OHG into mitochondrial DNA in neurodegenerative disease is known to elevate oxygen metabolism and contribute to mitochondrial failure. Such damage will ultimately decrease energy supplies and directly impact protein translation and degradation.

Oxidative radicals also impair transcription factor activity. Oxidized cysteine residues on zinc finger domains can disrupt the zinc coordination of transcription (74). Moreover, other metals such as aluminum and lead interfere with transcription by activating transcription factors that can induce cell death (54). When the regulation of pre-mRNA splicing is impaired by oxidative stress, the ratio of splice variants may shift which could increase the production of damaging transcripts. In addition, abnormal RNA editing of the GluR2 subunit of the AMPA receptor has been seen in amyotrophic lateral sclerosis, Huntington's and Alzheimer's diseases, and could contribute to excessive calcium permeability and cell death (2, 65).

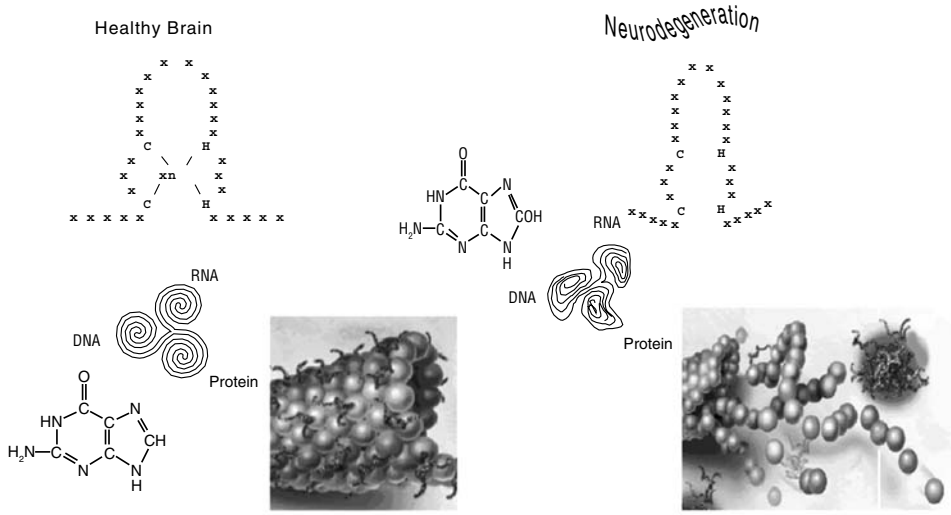

FIG. 1. The 'triskele' of gene expression in a healthy and neurodegenerative brain after exposure to oxidative stress. The triskele symbol represents three legs or branches radiating from a common center. In the case of gene expression, the phenotype of an individual (*the center*) is a product of the state (healthy or defective) of DNA, RNA and proteins (*the branches*). Each leg of expression is dependent on the others, creating an interdependent flow of information. In a healthy brain, nucleic acid damage is repaired, transcription and factors required for post-transcriptional processing function properly, and appropriate protein modifications and interactions are present. Overall, cellular homeostasis is maintained through normal physiological functions. In neurodegeneration, any branch may become distorted, resulting in the three branches of gene expression no longer being interdependent. Nucleic acids are improperly modified, transcription is disrupted, and protein processing spins out of control ultimately leading to the death of the neuron.

Protein metabolism is negatively impacted by oxidative stress. Damage to mitochondria, either through DNA damage or a dysfunctional Complex I will deplete the cell of its energy. The loss of ATP may shift proteolytic functions away from the energy-expensive UPS, to lysosomes, where proteins and their oxidized or nitrated residues accumulate. Increased cellular burdens of abnormal transcriptional or alternatively spliced gene products will only increase the proteinaceous deposits in lysosomes. The rapid accretion of lipid and protein residues into lipofuscin granules in neurodegenerative disease is, therefore, an important part of disease pathology and progression (45, 69). Ultimately, interference with cell homeostasis by oxidative radicals dysregulates gene expression and protein metabolism, and neuronal death ensues when the common upstream molecular processes contribute negatively to an already overburdened proteolytic system.

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ABBREVIATIONS

AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; cDNA, copy deoxyribonucleic acid; CRE, cyclic AMP response elements; CREB, CRE binding protein; DLB, dementia with Lewy bodies; DNA, deoxyribonucleic acid; MEF2, myocyte enhancer factor 2; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mRNA, messenger ribonucleic acid; MSA, multiple system atrophy; NII, neuronal intranuclear inclusions; 8OHG, 8- hydroxyguanosine; polyQ, polyglutamine; RNA, ribonucleic acid; ROS, reactive oxygen species; SOD, superoxide dismutase; UPS, ubiquitin-proteasomal system.

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